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(54) Title: PROTEIN GLYCOSYLATION ASSAY

(57) Abstract

A method of detecting and/or measuring the terminal glycosylation of a protein by estimating, preferably using labelled lectins, the relative proportions of different terminal sugars which may be present in the protein, is useful in providing a prognosis for various conditions, particularly breast cancer.

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PROTEIN GLYCOSYLATION ASSAY

This invention relates to the assay of glycosylated proteins and more particularly to detecting and/or measuring the terminal glycosylation patterns of proteins.

It is known that in some pathological conditions the terminal sugar residues of glycosylated proteins are altered. For example, it has been shown (Parekh et al, Nature 316 pp 452-457, 1985, and European Patent Publication No. 189388) that, in rheumatoid arthritis, serum IgG shows a reduced content of terminal galactose residues and a correspondingly increased content of terminal N-acetylglucosamine residues. The content of the latter in the protein is not increased, but the absence of galactose residues causes the N-acetylglucosamine residues to become the terminal sugar residues. It has also been shown (Leathem et al, The Lancet of May 9th, 1987 pp 1054-1057) that the prognosis for sufferers from breast cancer may be related to the ability of the cancer cells to bind a lectin specific for N-acetylgalactosamine residues. Cancer cells which bind this lectin were found to be associated with a propensity to metastasise to local lymph nodes. Conversely, cancer cells which did not bind to this lectin had a much lower tendency to form metastases. Other important carbohydrate alterations, detectable with lectins, occur in spina bifida, cystic fibrosis and prostatic cancer. Such alterations can also be significant

in the detection of (sometimes rare) blood groups, and changes in acute phase proteins in inflammation.

It is therefore of interest to be able to determine in a simple, cheap and rapid way the glycosylation patterns of proteins such as IgG and cell surface proteins. In practice, the terminal sugar residues likely to be of interest in many cases are those already noted, namely galactose (or N-acetyl-galactosamine) and N-acetylglucosamine (GlcNac). The former provides the usual terminal sugar residues in many cases, but the latter become the terminal residues when the glycosylation pattern of the protein in question is abnormal. In cystic fibrosis, terminal fucose residues are reduced with a corresponding increase in terminal N-acetyl-glucosamine residues.

The methods heretofore described for the detection and measurement of terminal glycosylation patterns have not been entirely satisfactory more particularly because they are complicated and expensive to operate. Thus Parekh et al used a method based on chromatography of oligosaccharides isolated from the IgG of rheumatoid arthritis patients on an agarose column to which the lectin from Ricinus communis was bound, or an enzymatic method. The method of Leathem et al involved incubating sections of cancer tissue with the lectin from the snail Helix pomatia (which binds to N-acetyl galactosamine residues), and adding rabbit anti-serum to this lectin

followed by biotinylated swine anti-rabbit immunoglobulin, and avidin-peroxidase. Peroxidase activity was then revealed using hydrogen peroxide and diamino benzidine. These methods are not simple.

We have now devised a simple means for detecting and/or measuring the glycosylation pattern of the terminal sugar residues of the oligosaccharide of a protein. The new method is rapid and simple to operate and uses readily available reagents. It is essentially based on the idea that what is required is not an absolute measure of the content of a particular possible terminal sugar residue, but rather an estimate of the relative proportions of the different terminal sugars which may potentially be present.

The method of the invention for detecting and/or measuring the terminal glycosylation pattern of a protein comprises treating samples of said protein of known comparability, which have been treated if necessary to expose terminal sugar residues, with a corresponding number of labelled reagents each having specific binding affinity for a particular terminal sugar residue which may be present in said protein, each such reagent being used in excess, removing unbound labelled reagent from the said samples, and then observing and/or measuring the signal provided by each bound or unbound reagent. By carrying out the method on samples of protein of known comparability, e.g. identical samples or samples which differ only in their dilution ratio, what is observed is the difference in

the ability of the samples to bind the labelled reagents which are chosen to be specific for the particular sugar residues to be determined. In principle it is possible to carry out the new method on a sample of the protein which is treated first with the labelled reagent and then (after removal of the first) with a second labelled reagent. In practice, however, it is preferred to use two samples simultaneously.

The preferred reagents having specific binding affinity for the terminal sugar residues are lectins. These are proteins of plant or animal origin which have specific binding affinity for particular sugar residues. Many lectins are known and they may be obtained commercially. They may be labelled by any of the methods customarily used for labelling proteins which are used in assay techniques based on specific binding affinities, e.g. by incorporation of radioactive atoms, or by binding, directly or indirectly, to enzymes. Enzymatic labelling is usually preferred as it avoids the need for the special equipment associated with the use of radioactive materials.

While the new method may be used in principle to study the terminal glycosylation pattern of any protein, it is especially useful for the study of proteins which lack a proportion of the usual terminal sugar residues. Thus, in the case of IgG, the new method is especially useful for measuring the proportion of molecules lacking the normal terminal galactose residues and having, as a consequence,

terminal N-acetylglucosamine residues. As noted above, the relative absence of terminal galactose residues is characteristic of the IgG of patients suffering from rheumatoid arthritis and the presence of terminal N-acetyl-galactosamine residues in the cell surface proteins of breast cancer tissue has been found to be associated with a tendency of the cancer to metastasise.

The new method is conveniently operated by making use of the ability of proteins such as IgG to bind strongly to certain solid substrates, especially nitrocellulose. Other solid substrates can be based on nylon (especially nylon-66, available from Amersham) and specially treated paper, especially amino-thiophenol treated paper available as Transbind APT paper. Suitable samples of the protein to be examined, after an appropriate purification treatment, are bound to the solid substrate. Sometimes it may be necessary to denature proteins either by boiling after binding to the solid phase or by disulphide bond reduction and treatment with 8M urea before binding to the solid phase. For human IgG, denaturation appears to be essential.

The bound samples are then treated with labelled lectins chosen for their specificity to the terminal sugar residues whose presence or absence is to be detected. The signals provided by the labels are then observed or measured, usually after appropriate development, e.g. with a colour forming reagent which is activated by the enzyme

used as label, and the ratio of the signals provided by the labelled reagents which react with, respectively, the normal terminal sugar residues and the abnormal terminal sugar residues, gives directly a measure of the latter. It should be noted in this connection that an absolute determination of the content of a particular terminal sugar residue is not required. All that is necessary is that the reagents used should be calibrated against known standards and that the samples of protein bound to the solid support should all be the same or have a known relationship to one another in terms of the quantity of protein.

The labelled lectins used in the new method may readily be prepared from commercially available lectins and commercially available labelling reagents. Suitable reagents are mentioned in the detailed description of the new method given below, but it will be understood that these reagents may be replaced by other reagents fulfilling the same function.

In the currently preferred method for operating the invention, IgG is isolated from blood samples in known manner and made up into a solution of known IgG content. This solution is then dot blotted onto a nitrocellulose sheet in carefully controlled quantity. The IgG binds tightly to the nitrocellulose. For dot blotting the samples, commercial dot blotters can be used. The assay may also be used on mixtures of proteins run on SDS-PAGE gels and western blotted onto nitrocellulose paper. The

blots can then be treated in the manner described herein. In that way, a range of proteins can be examined simultaneously. The method can however be applied to sugar estimation, using lectins or antibodies, using any type of reaction in a liquid medium or any type of solid phase support such as plastic microtitre plates, for example polystyrene ELISA plates such as those sold by Nunc or Dynatech.

In the preferred method, the dot blotted IgG is treated with a buffer containing a wetting agent and an inert protein, e.g. bovine serum albumin, to prevent further non-specific binding of protein to the nitrocellulose. The dots are then treated with the labelled lectin. The lectin may be, for example, biotinylated by reaction with N-hydroxysuccinimidobiotin. After washing to remove unbound lectin, the dots are treated with avidin having peroxidase (or other suitable enzyme) bound thereto. After further washing, the peroxidase, or other enzyme label, is detected by reaction with a suitable colour forming reagent, e.g. a combination of chloro-naphthol and hydrogen peroxide. The colour produced may be measured with an optical densitometer of the kind used in known enzyme linked immunoassay analysis. Alternatively the avidin may be labelled with a radio-isotope e.g. as ^{125}I-streptavidin. The bound radioactivity may then be measured directly.

Lectins from Abrus precatorius (abrin) and Ricinus communis (ricin; RCAI or RCAII B chain), are

suitable for binding to terminal galactose residues, while the lectins from Bandeiraea simplicifolia II (Griffonia simplicifolia II), Datura stramonium, or Lycoperiscon esculentum or succinylated wheat germ agglutinin, are suitable for binding to terminal N-acetylglucosamine residues. The Bandeiraea simplicifolia II (Griffonia simplificolia II) lecin is of particular interest because it is the only known lectin that interacts solely with terminal non-reducing - and - N-acetylglucosamine residues. Lectins from Lotus tetragonolobus, Griffonia simplicifolia IV and Ulex europaeus I bind to terminal fucose residues.

The following Examples illustrate the invention.

EXAMPLE 1

IgG purification

Blood samples were incubated at 37°C for one hour to allow clotting. They were then centrifuged at 3000 rpm for 25 minutes and the serum was removed and stored at -20°C. IgG was isolated from the serum (0.5 to 2 ml) in the following way. All the operations were carried out at room temperature. Half a volume of saturated ammonium sulphate solution at pH 7.2 was added dropwise with stirring to one volume of serum and the mixture was allowed to stand for 25 minutes and then centrifuged at 3000 rpm for 25 minutes. The precipitated protein pellet was resuspended in the original serum volume of 40% ammonium

sulphate solution, and then recentrifuged at 3000 rpm for 25 minutes. The protein pellet obtained was again suspended in the original serum volume of 20 mM potassium phosphate buffer at pH 7.2 (KPi). The solution obtained was dialysed against 20 mM KPi and then purified on a column of DEAE anion exchange cellulose (DE52, N about 8 g of gel in a 5 ml syringe barrel equilibrated with 20 mM KPi at pH 7.2; 2-5 g DEAE cellulose/ml of serum). The dialysed IgG solution (0.5 - 2 ml) was added to the column and eluted with KPi. The following fractions were collected:

(1)	2 ml
(2)	1 ml
(3)	4 ml
(4)	1 ml
(5)	5 ml

The IgG elutes in fractions (2) and (3). (This was checked by spectrophotometric examination at 280 nm.) The IgG-containing fractions were pooled and stored at 0-4°C in the presence of 0.01% of sodium azide. Protein concentration was estimated using Bradford Reagent (from Biorad). Each sample was checked for purity by electrophoresis on 10% SDS - polyacrylamide gel stained with Coomassie blue.

Assay procedure

The IgG samples purified in the above manner were diluted with phosphate buffered saline (hereinafter PBS, pH 7.2, containing 8 g NaCl, 0.2 g KCl, 0.2 g potassium

dihydrogen orthophosphate and 1.15 g dipotassium hydrogen orthophosphate/litre) to concentrations of 20 μg IgG and 40 μg IgG per 100 μl. A nitrocellulose membrane (Schleicher and Schuell, 0.1 μm pores) was prepared with a pencil grid (0.8 X 0.8 cm squares) and a suitable border for handling and then placed on twelve layers of absorbent tissue. The IgG samples were dot blotted onto the nitrocellulose. 5 μl of each IgG sample was applied using a 10 μl Hamilton syringe and Chaney adaptor. The application was arranged so that the needle of the syringe does not touch the nitrocellulose. The solution was carefully applied to reduce lateral movement of the solution along the membrane so that it was caused to pass through the nitrocellulose into the absorbent tissue. Each sample (including standards used for comparison) was applied in triplicate using 1 μg IgG/5 μl when abrin or ricin was used as the lectin probe and 2 μg IgG/5 μl when Bandeiraea lectin was used. The dot blots were then dried at room temperature for one hour after which they were boiled in PBS for five minutes to denature the IgG molecule and expose the sugar residues. The nitrocellulose membrane was then treated for one hour at room temperature (or at 0-4°C overnight) with 1% bovine serum albumin (BSA)in PBS containing 0.05% Tween wetting agent. (The bovine serum albumin reduces non-specific protein:protein interactions and the Tween wetting reagent reduces non-specific background binding to the nitrocellulose.)

The lectin probes were prepared by reacting each lectin with N-hydroxysuccinimidobiotin. The lectin in a concentration of 1 mg/ml in distilled water was treated with the biotinylating reagent at a strength of 1 mg/ml in dimethylsulphoxide in a ratio of biotin:lectin of 1:10 and left overnight at room temperature. The biotinylated lectin obtained was stored at 0-4°C in the presence of 0.01% of sodium azide.

The nitrocellulose membrane carrying the dot blots was incubated for two and a half hours with the biotinylated lectin. [The ricin-based reagent, RCAII B chain, was used at a dilution of 1/250, the abrin-based reagent at 1/125, and the Bandeiraea lectin based reagent at 1/100, all diluted with PBS containing BSA and Tween.] The blots were then washed five times at ten minute intervals with PBS containing Tween and BSA. They were then incubated for two hours on a rotary shaker with Streptavidin labelled with Horse Radish Peroxidase at 1/1000 in PBS-Tween-BSA. The blots were washed five times at ten minute intervals with PBS containing Tween only, and then developed with 4-chloro-naphthol and hydrogen peroxide. To one volume of methanol was added 0.3 mg/ml of 4-chloro-1-naphthol followed by five volumes of Tris buffer (pH 7.6) and 0.01% of hydrogen peroxide (0.1% for Bandeiraea lectin treated blots). The mixture was prepared immediately before use. After the colour had formed, the membrane was washed with distilled water and the blots were

dried thoroughly. Their densities were then read with a photometer.

Figure 1 shows the relationship between absorbance measured with the photometer and percentage oligosaccharide chains lacking galactose. The latter were obtained by quantitative biochemical analysis using the method described by Dwek et al (loc. cit.) The excellent correlation between the new method and the Dwek et al method is evident. Figure 2 shows a plot of the absorbance ratio of dots measured using Bandeiraea lectin to dots measured using ricin, i.e. a measure of the ratio of terminal N-acetylglucosamine residues to terminal galactose residues. It will be noted that the new method provides a very sensitive measure of the percentage of oligosaccharide chains lacking galactose in the range 20-60%.

The dot blot assay can be modified and performed on polystyrene ELISA plates as illustrated in Example 2.

Example 2

The purified samples of human IgG to be analysed were treated with 8M urea containing 0.5M mercaptoethanol (Sample/urea ratio = 2mg/2ml) and incubated overnight at 37°C. The treated samples were then dialysed overnight against 0.02M iodoacetamide, followed by dialysis against PBS for 2h. This first step of treating samples with urea is only necessary if the samples need to be denatured to expose the carbohydrates. A human IgG sample is believed to require denaturation whereas this does not appear to be necessary for a mouse IgG sample.

Protein estimation was carried out on the denatured human IgG samples and un-denatured mouse IgG samples. Elisa plates were coated with the sample (100μl of sample per well) at a protein concentration of 10μg/ml in carbonate/bicarbonate buffer, pH 9.6. The plates were left at 4^{0}C overnight or 3h at room temperature after which they were washed three times with PBS followed by blocking with PBS-Tween (0.05%)-BSA (1%) for 1h at room temperature. After washing three times with PBS, the biotinylated reagents (100μl/well) used in the dot blot assay of Example 1 were added and the plates incubated overnight at 4°C.

The diluent for the reagents was PBS-Tween-BSA 0.1%.

The plates were washed again 3 times with PBS-Tween and streptavidin-HRP conjugate (100μl/well) added. The plates were incubated for 2h at room temperature followed by washing three times with PBS-Tween.

Substrate was then added to the wells and the colour developed in the dark for 10-30 minutes. The reaction was stopped using sodium fluoride (96mg/50ml $H_2 0$) and the plates read at 405nm.

The substrate used was 2'2' Azino-di(3,ethyl benzthiazoline sulfonic acid in 0.05M citrate phosphate buffer, pH4.1. 50mg substrate was present in 100ml citrate phosphate buffer + 35μl $H_2 0_2$ vol 20.

The results obtained for human and mouse IgG samples are given in Table 1.

Table 1

a) Analysis of human IgG glycosylation using an Elisa assay

%Go	Binding to Galactose	Binding to GlcNac	Ratio of Gal binding/ GlcNac binding
21	0.677	0.392	0.78
32	0.509	0.643	1.18
38	0.430	0.750	2.25
61	0.107	0.472	6.27

The degree of galactose deficiency (Go) is shown to be related to the ratio in the final column and this method of measuring the terminal glycosylation pattern is believed to be particularly sensitive and versatile.

b) Mouse IgG

Untreated IgG Sample (Strain of mouse)	Binding to GlcNac
SJL	0.074
Balb/c	0.268
MRL ++	0.386
MRL Ip	0.515

The GlcNac residues on mouse IgG bound to a plastic solid phase are essentially exposed to anti-GlcNac without the need for prior denaturation.

CLAIMS

1. A method for detecting and/or measuring the terminal glycosylation pattern of a protein comprising treating samples of said protein of known comparability, which have been treated if necessary to expose terminal sugar residues, with a corresponding number of labelled reagents each having specific binding affinity for a particular terminal sugar residue which may be present in said protein, each such reagent being used in excess, removing unbound labelled reagent from the said samples, and then observing and/or measuring the signal provided by each bound or unbound reagent.

2. A method according to claim 1 in which the reagents each having specific binding affinity for a terminal sugar residue are lectins.

3. A method according to claim 2 in which the lectins are from Abrus precatorius, Ricinus communis, Bandeiraea simplicifolia, Datura stramonium or Lycoperiscon esculentum.

4. A method according to any one of claims 1 to 3 in which the terminal sugar residue is galactose or N-acetylglucosamine.

5. A method according to any one of claims 1 to 4 in which the reagents each having a specific binding affinity for a terminal sugar residue are each enzyme labelled.

6. A method according to any one of claims 1 to 5 in which the protein to be examined is IgG.

7. A method according to any one of claims 1 to 6 in which the samples of said protein to be examined are bound to a solid substrate prior to treatment with the labelled reagents.

8. A method according to claim 7 in which the solid substrate is nitrocellulose.

9. A method according to claim 7 in which the solid substrate is a polystyrene.

10. A kit suitable for carrying out the method of any one of claims 1 to 9, comprising a solid substrate onto which the protein may be bound, and a number vials each containing a labelled reagent having a specific binding affinity for a particular terminal sugar residue.

Fig. 1.

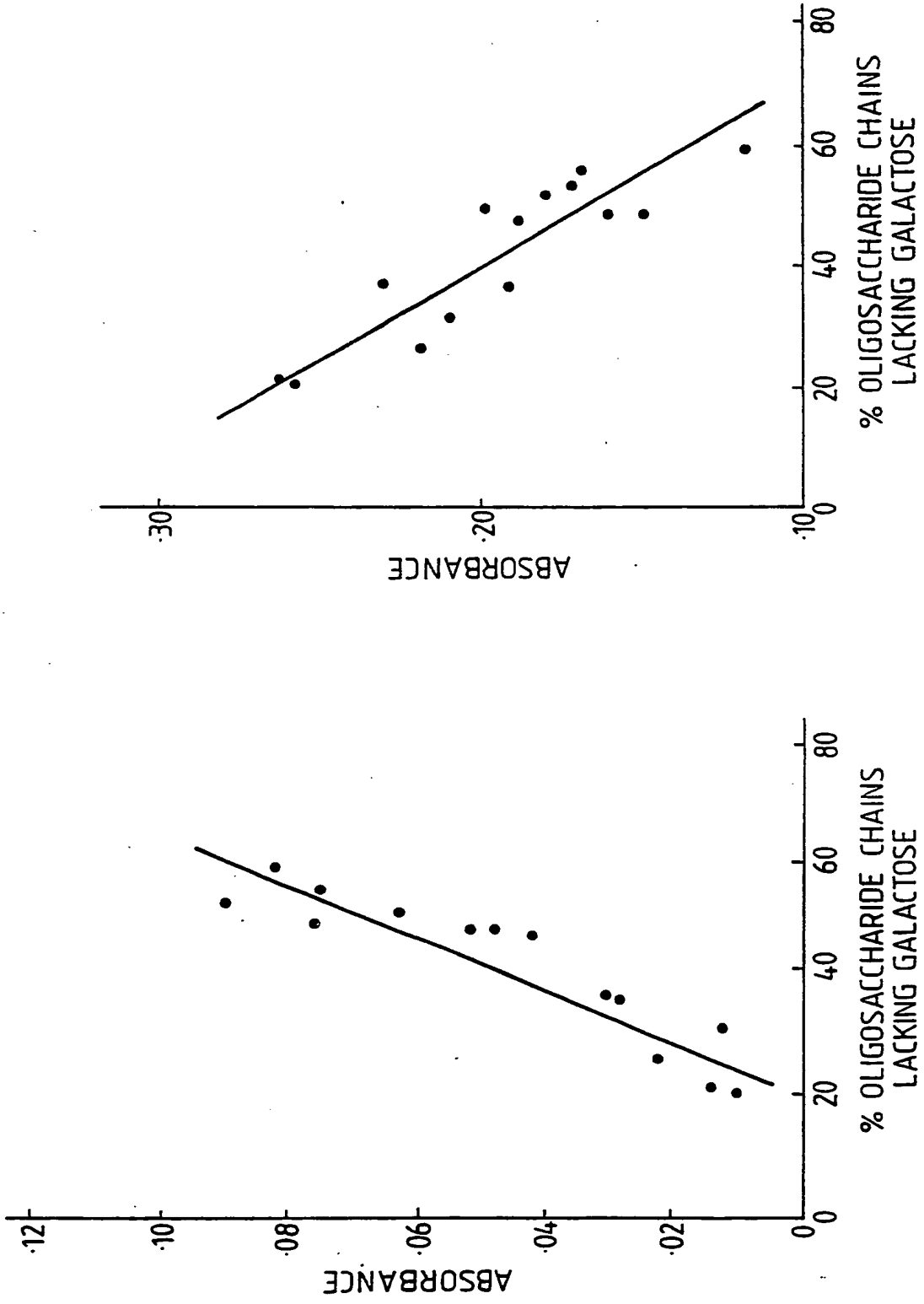
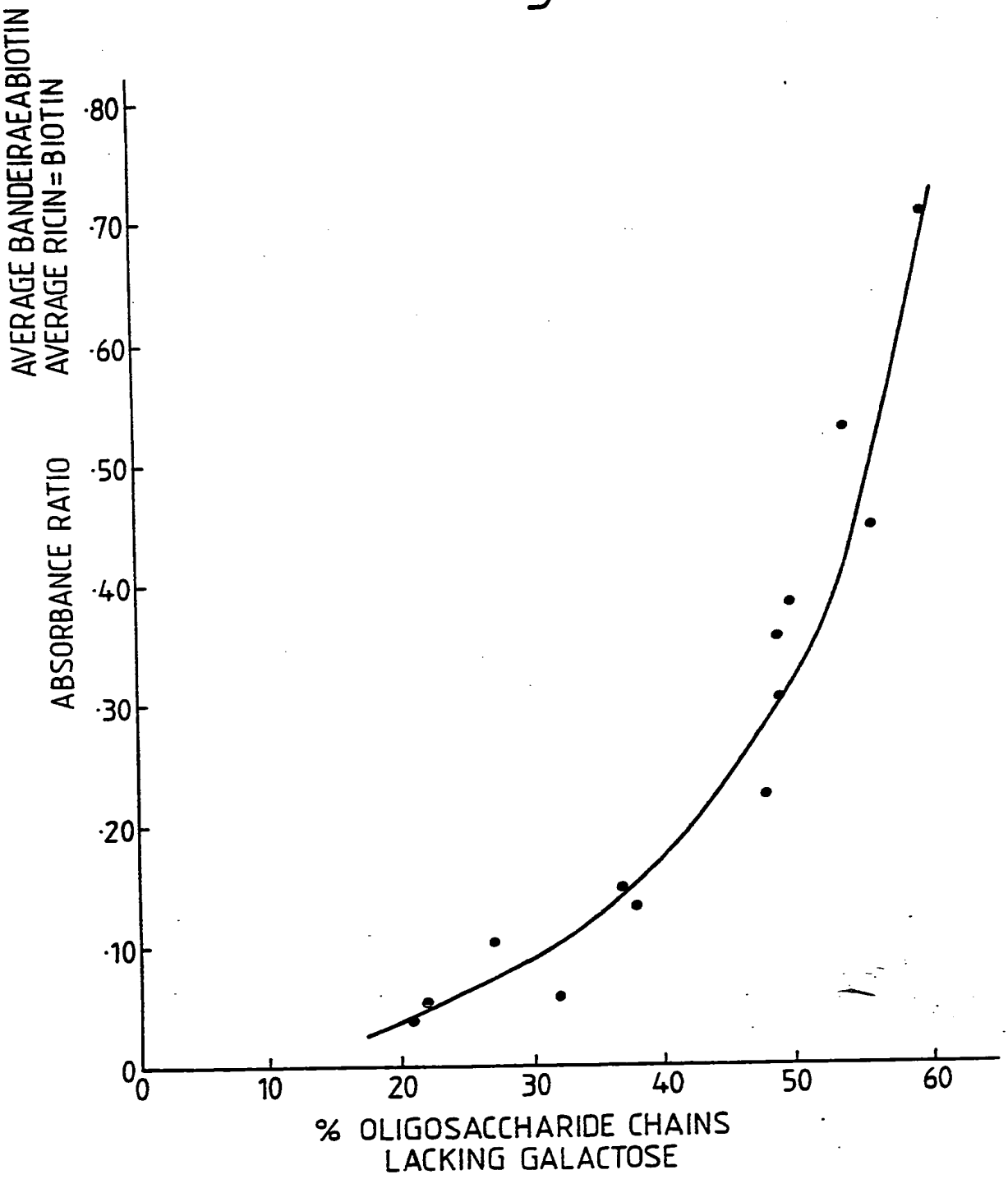

AVERAGE
RICIN-BIOTIN (1μg)
ABSORBANCE
·30
·20
·10
0
20
40
60
80
% OLIGOSACCHARIDE CHAINS
LACKING GALACTOSE
AVERAGE
BANDEIRAEABIOTIN (2μg)
ABSORBANCE
·12
·10
·08
·06
·04
·02
0
20
40
60
80
% OLIGOSACCHARIDE CHAINS
LACKING GALACTOSE

Fig. 2.


AVERAGE BANDEIRAEABIOTIN
AVERAGE RICIN=BIOTIN
ABSORBANCE RATIO
·80
·70
·60
·50
·40
·30
·20
·10
0
0
10
20
30
40
50
60
% OLIGOSACCHARIDE CHAINS
LACKING GALACTOSE

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 88/00978

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: G 01 N 33/68; G 01 N 33/544; // G 01 N 33/574; G 01 N 33/80; G 01 N 33/564

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0057236 (OTSUKA PHARMACEUTICAL CO. LTD) 11 August 1982 see page 1, line 1 - page 8, line 7; page 11, line 9 - page 14, line 6; claims	1-5,10
X,P	EP, A, 0255342 (UNIVERSITY COLLEGE LONDON) 3 February 1988 see the whole document	1,4-8,10
X	Chemical Abstracts, volume 108, no. 7, 15 February 1988, (Columbus, Ohio, US), R. Lotan et al.: "Enhanced glycosylation of a melanoma cell surface glycoprotein by retinoic acid: carbohydrate chain analysis by lectin binding", see page 610, abstract 54738p, & Cancer Biochem. Biophys. 1987, 9(3), 211-21	1-3,7
X	Chemical Abstracts, volume 104, no. 7, 17 February 1986, (Columbus, Ohio, US), ./.	1-3,7,8

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th February 1989	29 MAR 1989
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: P.C.G. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No. PCT/GB 88/00978

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8800978 SA 25387

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 13/03/89
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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